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Rapid Report

Large-scale preparation of pure plastocyanin from spinach

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50 mg of pure plastocyanin was prepared from 5 kg spinach leaves in four steps: (1) acetone extraction; (2) ammonium sulfate fractionation; (3) Sepharose column chromatography; and (4) concentration. The acetone and ammonium sulfate steps allowed for the use of a single chromatographic step which yielded plastocyanin free of contaminants.

Plastocyanin functions between cytochrome *f* and Photosystem I in the photosynthetic electron transport chain [1]. Our studies on the interaction between plastocyanin and cytochrome *f* required large quantities of pure plastocyanin for cross-linking experiments and kinetic analyses. We had been relying on the methods of Ellefson et al. [2] or Ashton and Anderson [3] for the preparation of plastocyanin. The method of Ellefson et al. employs an acetone extraction of homogenized plant tissue at the beginning of the plastocyanin preparation. This is followed by ammonium sulfate fractionation and then repeated ion-exchange chromatography to obtain pure plastocyanin. Acetone efficiently extracts membrane components such as lipids, carotenoids and chlorophyll which interfere with column chromatography and the visible detection of plastocyanin. The acetone and ammonium sulfate steps also removes nearly all other proteins. A very different protocol is used by Ashton and Anderson. In this procedure the plant tissue is homogenized in a solution containing Triton and ammonium sulfate. After a filtration step the mixture is brought to a higher ammonium sulfate concentration. Plastocyanin, which remains soluble in the high concentration of ammonium sulfate, is collected on a Sepharose column in the presence of ammonium sulfate and eluted in the absence of ammonium sulfate. Pure plastocyanin is then obtained by ion-exchange chromatography. This last chromatographic step is reported to remove a substance which absorbs ultraviolet light at 278 nm from an otherwise pure preparation of plastocyanin. The distinct advantage of this method over that of Ellefson et al. is that plastocyanin protein is purified by essentially one chromatographic step. However, when we employed the method of Ashton and Anderson, the green and very viscous homogenate resulting from the mix of plant material, detergent and ammonium sulfate interfered with the smooth execution of the filtration and chromatographic steps. The filtration rate would become unacceptably slow and frequent changes of the filtration paper was required. Also, after one chromatographic step, the Sepharose matrix could not be reused and was discarded. Our yields of plastocyanin were consequently, variable and lower than the yields obtained by the method of Ellefson et al. We combined the two procedures by utilizing the acetone extraction and ammonium sulfate fractionation steps in the Ellefson et al. protocol and the Sepharose column chromatography procedure of Ashton and Anderson. The ammonium sulfate fractionation represents the cross-over point between the two parent methods. Thus, we were able to include the advantages and eliminate the disadvantages from both methods. The procedure we used is described below.

The entire procedure was performed at 4–10°C. One bushel of market spinach was washed and the leaves were removed from the stems. The leaves (approximately 5 kg) were homogenized with a large blender in 6 L of 0.4 M sucrose, 50 mM KCl, 50 mM Tris-HCl (pH 8.0), containing 0.2 ml antifoam A (Sigma) per liter. When antifoam A was omitted, 10 L of the homogenization solution was required. The homogenate was filtered through four layers of cheese cloth. The leaf pulp retained on the cheese cloth was squeezed by hand to remove more of the aqueous extract. The remaining pulp was discarded. Chloroplasts were collected by centrifugation at $10\,000 \times g$ for 10 min. The chloroplasts pellets were resuspended to

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final volume of 400 ml with 500 mM Tris-HCl (pH 8.0). Cold acetone (−15°C) was added slowly with vigorous stirring to the chloroplast suspension to a final concentration of 35% (v/v). The mixture was stirred an additional 15 min. and then centrifuged at 10000 × *g* for 10 min. The pellet was discarded and cold acetone (−15°C) was added slowly with vigorous stirring to the supernatant fraction bringing the final concentration of acetone to 80% (v/v). The precipitate was allowed to settle and after 1 h most of the liquid could be poured off. The remaining liquid and precipitate were centrifuged at 10000 × *g* for 10 min. The protein pellet was resuspended in 200 ml of 50 mM potassium phosphate (pH 7.4). 1 g of Bio-Rad AG1-X8 resin (100–200 mesh) was added to the suspension to absorb phenolic substances. The suspension was dialyzed overnight against 10 L of 20 mM potassium phosphate (pH 7.4) in 6000–8000 molecular weight cut-off dialysis tubing (Spectrapor).

Solid ammonium sulfate (200 g/L) was added slowly with stirring to the dialyzed suspension. Precipitated material and residual resin were removed by centrifugation at 10000 × *g* for 10 min. Solid ammonium sulfate (300 g/L) was added slowly with stirring to the supernatant solution. Precipitated material was removed by centrifugation at 10000 × *g* for 20 min. Approx. 5 to 20 mg of potassium ferricyanide were added to the supernatant fraction. The supernatant fraction was passed through a column of Sepharose 4B (2.5 cm × 20 cm) which was equilibrated with 2.9 M ammonium sulfate, 10 mM KCl, 50 mM Tris-HCl (pH 7.4). Plastocyanin was absorbed onto the column and its blue color was visible. The column was washed with 100 ml (one column volume) of the equilibration solution. Plastocyanin was eluted with 10 mM KCl, 50 mM Tris-HCl (pH 7.4) plus an added 5 to 20 mg potassium ferricyanide.

The eluted plastocyanin was concentrated with an Amicon ultrafiltration unit utilizing a YM10 membrane. Concentrated plastocyanin was dialyzed twice against 2 L of 5 mM KCl, 5 mM potassium phosphate (pH 7.4) in a 6000–8000 molecular weight cut-off dialysis tubing. Alternatively, dialysis was performed during the concentration step by repeated dilution of the concentrate. Protein concentration determined by the BCA protein assay (Pierce) agreed with the spectral assay for the concentration of plastocyanin (A_{597nm}, oxidized − reduced, $e_{mM} = 4.5$) [4]. Typical yields were 30 to 50 mg of pure plastocyanin per 5 kg spinach leaves. SDS-PAGE analysis [5] revealed a single band by staining the gel with Coomassie blue. Thus, plastocyanin was judged to be pure by these results taken collectively.

This method proved to be very reproducible and was easily scaled up or down to handle different quantities of material. There was no interference with spectral readings of plastocyanin at 278 nm as reported by Ashton and Anderson. The reported interfering substance may have originated from the detergent Triton or from plant phenolic compounds. In the procedure described by this report Triton was not used and any phenolic compounds were removed prior to the elution of plastocyanin from the Sepharose column. During the washing of the Sepharose column, plastocyanin moved slowly down the column. However, its migration was substantially slower than the flow rate of the wash solution and it remained near the top of the column. The Sepharose matrix was reusable through multiple preparations. Plastocyanin can be used directly from the Sepharose column or after concentration by ultrafiltration depending upon the purpose. Dialysis is recommended since the solution will have a defined composition. Plastocyanin prepared by this method was used for cross-linking studies with cytochrome *f* using the chemical agent 1-ethyl-3-(3-dimethyl)aminopropyl carbodiimide [6]. Thus, it was essential that the Tris buffer and residual ammonium sulfate, which would interfere with the cross-linking reaction, be removed from the plastocyanin preparation. If an ultrafiltration apparatus is not available, then plastocyanin can be concentrated by ion-exchange chromatography [2,3].

Large amounts of pure plastocyanin would be valuable for many ongoing and possible future researches involving plastocyanin and the interaction with its photosynthetic reaction partners. For example, the recent crystalization of Photosystem I [7] and cytochrome *f* [8] has created the possibiltiy that plastocyanin could be co-crystalized with its reaction partners. This procedure will allow for the easy preparation of large quantities of purified plastocyanin suitable for such purposes.

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